the virus in embryonated chicken eggs. Not all influenza virus strains replicate well in eggs; thus the viruses must be adapted or viral reassortants constructed. Extensive heterogeneity occurs in the hemagglutinin of egg-grown influenza viruses as compared to primary isolates from infected individuals grown in mammalian cells (Wang, et al. *Virol.* 171:275–279 (1989); Rajakumar, et al. *Proc. Natl. Acad. Sci. USA* 87:4154–4158 (1990)). The changes in HA during the selection and manufacture of influenza vaccines can result in a mixture of antigenically distinct subpopulations of virus. The viruses in the vaccine may therefore differ from the variants within the epidemic strains, resulting in suboptimal levels of protection.

Immediate hypersensitivity reactions can occur in persons with severe egg allergy due to residual egg protein in the vaccine. The 1976 swine influenza vaccine was associated with an increased frequency of Guillain-Barré syndrome. Subsequent vaccines prepared from other influenza strains have, thus far, not been observed to increase the occurrence of this rare disease.

A method of producing an influenza vaccine that does not require propagation in eggs would result in a purer product that would be less likely to cause an adverse immune reaction. In addition, a purer vaccine preparation would not require virus inactivation or organic extraction of viral membrane components, thereby avoiding denaturation of antigenic epitopes and safety concerns due to residual chemicals in the vaccine.

In addition, an influenza vaccine produced in the absence of egg propagation would avoid the genetic heterogeneity that occurs during adaptation and passage through eggs. This would result in a vaccine that is better matched with influenza epidemic strains, resulting in improved efficacy.

It is therefore an object of the present invention to provide a method of producing an influenza vaccine that does not require replication in eggs.

It is a further object of the present invention to provide a method of producing an influenza vaccine that is rapid and cost-efficient, highly purified and allows production of vaccines from primary sources of influenza.

## SUMMARY OF THE INVENTION

A method of preparing a recombinant influenza hemagglutinin protein by expression in insect cells using a baculovirus expression system is provided. The resulting protein is useful in making vaccine consisting of a trivalent influenza vaccine based on a mixture of recombinant hemagglutinin antigens cloned from influenza viruses having epidemic potential. The recombinant hemagglutinin proteins are full length, uncleaved (HA0) glycoproteins purified under non-denaturing conditions to 95% or greater purity. The recombinant HA0 glycoproteins can be cleaved at the disulfide bond to form the two chains, HA1 and HA2.

A process for cloning influenza hemagglutinin genes from influenza A and B viruses using specially designed oligonucleotide probes and polymerase chain reaction (PCR) methodology is also disclosed. The cloned HA genes are modified by deletion of the natural hydrophobic signal peptide sequences and replacement with a new baculovirus signal peptide. These chimeric genes are introduced into baculovirus expression vectors so that the baculovirus polyhedrin promoter directs the expression of recombinant HA proteins in infected insect cells. The 18 amino acid baculovirus signal peptide directs the translation of rHA into the insect cell glycosylation pathway and is not present on the mature rHA glycoprotein.

This methodology can be extended to all types of influenza viruses, including but not limited to the prevalent A (H1N1) sub-type, the A(H3N2) sub-type, and the B type that infect humans, as well as the influenza viruses which infect other mammalian and avian species.

A general approach for the efficient extraction and purification of recombinant HA protein produced in insect cells is disclosed which can be adapted for the purification of rHA proteins from A sub-types and B type influenza viruses. The recombinant vaccine can be developed from primary sources of influenza, for example, nasal secretions from infected individuals, rather than from virus adapted to and cultured in chicken eggs. This allows rapid development of vaccine directly from epidemic strains of influenza and avoids the problems arising from adaptation of the virus for culture in eggs, as well as patient reaction to egg contamination in the resulting vaccine. In one embodiment, the vaccine is formulated in an immunizing dosage form including purified rHA antigens from three strains of influenza virus recommended by the FDA for the 1993/94 influenza epidemic season. Functional immunity can be measured using assays that quantitate antibodies that bind to influenza hemagglutinin, that block the ability of influenza virus to agglutinate red blood cells, or that neutralize the influenza virus. Protective immune responses with rHA vaccines can also be measured in animals that are susceptible to influenza infection or in human challenge studies.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a schematic of the cloning of HA genes from influenza A strains from purified viral RNA preparations, purification of expressed rHA, and biological characterization of rHA. Abbreviations: FDA, Food and Drug Administration; MDCK, Madin Darby Canine Kidney; TPCK, tosylphenylalanylchloromethylketone; RNA, ribonucleic acid; cDNA, complementary deoxyribonucleic acid; HA, hemagglutinin; FBS, Fetal Bovine Serum; PCR, Polymerase Chain Reaction; and BV, Baculovirus.

FIG. **2** is a more detailed schematic of the method of FIG. **1** applied to the cloning and expression of the HA gene of the Influenza A/Texas/36/91 strain. Influenza HA gene was obtained from RNA purified from MDCK cells infected with influenza A/Texas/36/91 using reverse transcriptase and a universal primer (SEQ ID NO.1) followed by two rounds of PCR amplification and cloning, wherein the 5' and 3' primers shown in the first round are set forth in SEQ ID NOs. 2 and 3, respectively, and wherein the 5' and 3' primers shown in the second round are set forth in SEQ ID NOs. 4 and 3, respectively. A baculovirus recombination vector was constructed containing the polyhedrin promoter and a signal peptide sequence from the baculovirus 61K gene (a baculovirus gene that encodes a signal peptide having a molecular weight of approximately 61,000), followed by the complete coding sequences for the mature HA protein. This recombination vector was then used to make a baculovirus expression vector that produces HA from this strain of the virus.

FIG. **3** is a graph of the anti-HA immune response in mice, day 42, n=5, graphing antibody titer for rHA0-neat; Fluzone® (licensed trivalent subvirion vaccine containing 15 μg/dose of each the HAs from influenza A/Texas/36/91 ($N_1N_1$), A/Beijing/32/92 ($H_3N_2$) and B/Panama, 45/90 viruses, i.e., 45 μg HA/ 0.5 mL dose; Connaught Laboratories, Inc., Swiftwater, Pa.) vaccine, and rHA0-alum, at dosages of 0.5 μg (dark bars), 0.1 μg (shaded bars), 0.02 μg (dotted bars), and 0.004 μg (open bars).